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# Lower prevalence but similar fitness in a parasitic fungus at higher radiation levels near Chernobyl

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## Abstract

Nuclear disasters at Chernobyl and Fukushima provide examples of effects of acute ionizing radiation on mutations that can affect the fitness and distribution of species. Here, we investigated the prevalence of *Microbotryum lychnidis-dioicae*, a pollinator-transmitted fungal pathogen of plants causing anther-smut disease in Chernobyl, its viability, fertility and karyotype variation, and the accumulation of nonsynonymous mutations in its genome. We collected diseased flowers of *Silene latifolia* from locations ranging by more than two orders of magnitude in background radiation, from 0.05 to 21.03  $\mu\text{Gy/h}$ . Disease prevalence decreased significantly with increasing radiation level, possibly due to lower pollinator abundance and altered pollinator behaviour. Viability and fertility, measured as the budding rate of haploid sporidia following meiosis from the diploid teliospores, did not vary with increasing radiation levels and neither did karyotype overall structure and level of chromosomal size heterozygosity. We sequenced the genomes of twelve samples from Chernobyl and of four samples collected from uncontaminated areas and analysed alignments of 6068 predicted genes, corresponding to  $1.04 \times 10^7$  base pairs. We found no dose-dependent differences in substitution rates (neither dN, dS, nor dN/dS). Thus, we found no significant evidence of increased deleterious mutation rates at higher levels of background radiation in this plant pathogen. We even found lower levels of nonsynonymous substitution rates in contaminated areas compared to control regions, suggesting that purifying selection was stronger in contaminated than uncontaminated areas. We briefly discuss the possibilities for a mechanistic basis of radio resistance in this nonmelanized fungus.

**Keywords:** bumblebees, butterflies, genomic degeneration, melanin, *Microbotryum violaceum*, positive selection, red pigment

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## Introduction

Natural levels of radioactivity on Earth vary by more than 1000-fold, and such spatial heterogeneity suffices

to create highly contrasting exposure rates to organisms across environmental conditions. Levels of background ionizing radiation (hereafter radiation) have decreased by a factor of ten since terrestrial life first originated in the pre-Cambrian (Karam & Leslie 2005). Fluctuations in natural levels of radiation due to solar flares, supernovae and gamma ray bursts, and large meteor impacts in Mexico, India, Russia and other sites likely caused

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the release of vast amounts of radioactive material on Earth (e.g. Alvarez *et al.* 1980). Such fluctuations may have contributed to current levels of resistance to radiation damage in free-living organisms. Today, typical background radiation dose rates vary from low values of only 0.01 to 0.10  $\mu\text{Sv/h}$ , with the natural level in Chernobyl before the nuclear accident being 0.01–0.03  $\mu\text{Sv/h}$  (Ramzaev *et al.* 2006). There are many high-radiation sites in the oceans, with thermal vents being a well-known example (e.g. Fiala-Médioni *et al.* 1986; Cherry *et al.* 1992; Jollivet *et al.* 1995). Maximum terrestrial levels of radioactivity reach 29.7  $\mu\text{Sv/h}$  in Ramsar, Iran (Ghiassi-Nejad *et al.* 2002).

Mutations are changes in genomic sequences of DNA that may occur as a consequence of imperfect repair of single or double strand breaks (Lehman 2006; von Sonntag 2010). Radiation was first shown to be a powerful mutagen in classical laboratory experiments almost a century ago (Nadson & Philippov 1925; Muller 1954; UNSCEAR 1988; National Academy of Sciences – Natural Resources Council. Committee on the Biological Effects of Ionizing Radiation. BEIR V. 1990). However, it is less well known how natural variation in levels of background radiation influence mutation rates in nature (e.g. Forster *et al.* 2002; meta-analysis in Møller & Mousseau 2013). Such naturally occurring mutations are an important source of novel genetic variation that forms the raw material for evolution (Hartl 1988), but both germline and somatic mutations may also cause genetic diseases, including cancer, and impose a ‘genetic load’ on fitness (Lynch & Gabriel 1990).

Fungi show extraordinary abilities to cope with ionizing radiation. For example, microfungi associated with thermal vents can live under extremely high-radiation levels (e.g. Shrivage *et al.* 2007; Charmasson *et al.* 2009). Several microfungi from irradiated areas are directly attracted by radionuclides (positive radiotropism), being able to grow on ‘hot particles’ and even degrade them (Zhdanova *et al.* 2004). Furthermore, ionizing radiation may have a positive stimulatory effect on spore germination (Tugay *et al.* 2006). Indeed, ionizing radiation promotes growth of some fungi that produce the polymer melanin, transferring electrons to melanin and potentially even exploiting redox properties to transduce energy for cell metabolism (Dadachova *et al.* 2007; Dadachova & Casadevall 2008). Thus, most radio-resistant fungi have been found so far to differ from non-radio-resistant species in their level of accumulation of intracellular melanin (Dadachova *et al.* 2007; Khajo *et al.* 2011; Tugay *et al.* 2011).

The nuclear accident at Chernobyl on 26 April 1986 was followed by research providing extensive evidence of somatic and germline mutations in plants (e.g. Kovalchuk *et al.* 2000) and animals (e.g. Ellegren *et al.* 1997).

However, because Chernobyl was a single event, comparing current Chernobyl populations with nonirradiated nearby populations is insufficient to attribute any difference to radiation. For statistical tests of the effect of radiation, one has either (i) to correlate traits with radiation levels in the field across a range of radiation severities or (ii) to show that a trait has changed in Chernobyl after the nuclear disaster but has not changed in nonradiated areas nearby (Møller & Mousseau 2006). Genetic damage from 45 studies of 30 species revealed a mean effect size of 0.67 measured as Pearson’s product-moment correlation coefficient, with radiation level accounting for 44.3% of the variation in mutation rate (Møller & Mousseau 2015). This effect size is one of the largest ever recorded in ecological sciences. Although there was consistency within species in effect size (i.e. significant repeatability), there was significant heterogeneity among species, with no evidence of phylogenetic signal nor any clear ecological predictors of effect size variation (Møller & Mousseau 2015).

The predicted effects of radiation exposure include chromosomal aberrations (Wang *et al.* 1990; Møller & Mousseau 2013), an increased substitution rate due to damaged DNA (Martincorena & Campbell 2015) and, as a probable consequence, an increased rate of deleterious mutations (Premi *et al.* 2009; Møller & Mousseau 2013). Karyotype analysis is one way to assess chromosomal damage. Several studies have reported chromosomal aberrations following exposure to high-radiation levels (Kochupillai *et al.* 1976; Wang *et al.* 1990; Chen & Wei 1991; Cheriyan *et al.* 1999; Jiang *et al.* 2000; Hayata *et al.* 2004; Møller & Mousseau 2013). Such changes in genome structure commonly lead to irregularities of chromosome pairing and segregation during the meiotic divisions, with consequences for the viability of the haploid, gametic stage of the life cycle (Gillies 1989). Computational analysis of genes and genomes of organisms exposed to excessive radiation, typically as a result of a nuclear disaster, such as those experienced in Chernobyl and Fukushima, is another way to reveal signatures of increased mutation rates (looking at dS, the synonymous substitution rate) or of amino-acid substitution rates (dN, the nonsynonymous substitution rate), the latter often having deleterious effects (Møller & Mousseau 2013, 2015). In addition, increased rates of deleterious substitutions (i.e. genomic degeneration) can be expected, in particular in organisms showing decreased effective population sizes due to high mortality rates in irradiated areas (Woolfit & Bromham 2005). Increased rates of deleterious substitutions can be detected by increased ratios of nonsynonymous to synonymous rates of substitutions, represented as dN/dS (Anisimova & Liberles 2012; Fontanillas *et al.* 2015). On

the other hand, selection may be stronger in irradiated areas if radiation is a source of physiological stress (Møller 1993, 2002; Ellegren *et al.* 1997; Møller & Mousseau 2001, 2003; Møller *et al.* 2005a,b, 2012, 2013), thereby purging deleterious mutations and reducing detected ratios of nonsynonymous to synonymous rates of substitutions (i.e. lower dN/dS ratios). Some cases of possible adaptation to radiated conditions in Chernobyl have even been reported (Møller & Mousseau 2016).

Although radionuclide accumulation has been investigated in fungi, in particular in edible and mycorrhizal fungi (Mascanzoni 2001; Mietelski *et al.* 2010; Gwynn *et al.* 2013), few studies have investigated the effect of contamination in Chernobyl on the abundance and fitness of fungi from the contaminated environments (Møller & Mousseau 2013, 2015), with a few exceptions (Tugay *et al.* 2006; Dadachova & Casadevall 2008). Certain fungi were found to cope very well with these high-radiation levels, some even thriving in the defunct Chernobyl nuclear reactor (Zhdanova *et al.* 2000; Dadachova & Casadevall 2008). Fungi are good models for studying genomic consequences of radiation because they have small genomes that can easily be fully sequenced (Gladieux *et al.* 2014), and some are easy to grow in vitro, allowing viability and fertility measures. In addition, they play important ecological roles as pathogens, mutualists or decomposers.

Here we investigated consequences of the Chernobyl disaster on a plant pathogenic fungus. *Microbotryum lychnidis-dioicae* is a fungus causing anther-smut disease on the dioecious white campion, *Silene latifolia*. The pathogen castrates the plant by producing its spores in place of pollen in anthers of male flowers while aborting ovaries and inducing spore-bearing anthers in female flowers. Spores are transmitted to healthy flowers by pollinators (Roche *et al.* 1995), and insect abundance has been shown to decrease dramatically with radiation level in Chernobyl (Møller & Mousseau 2009). *Microbotryum lychnidis-dioicae* is highly selfing, undergoing mostly intratetrad mating (Hood & Antonovics 2000; Giraud *et al.* 2005; Zakharov 2005), and is therefore highly homozygous (Giraud 2004; Vercken *et al.* 2010). *Microbotryum lychnidis-dioicae* is a model organism in ecology and evolution (Antonovics *et al.* 2002; Bernasconi *et al.* 2009), and a reference genome has been published recently (Badouin *et al.* 2015; Perlin *et al.* 2015). Previous studies based on microsatellite markers and gene sequences revealed a strong population structure in *M. lychnidis-dioicae* at the European scale, with three main clusters corresponding to glacial refugia, in Western Europe, the Italian peninsula and Central-Eastern Europe, respectively (Vercken *et al.* 2010; Gladieux *et al.* 2011). Therefore, we used fungal strains from the Central-Eastern European genetic cluster but not near

the Chernobyl area as control strains from noncontaminated areas to avoid biases due to population structure.

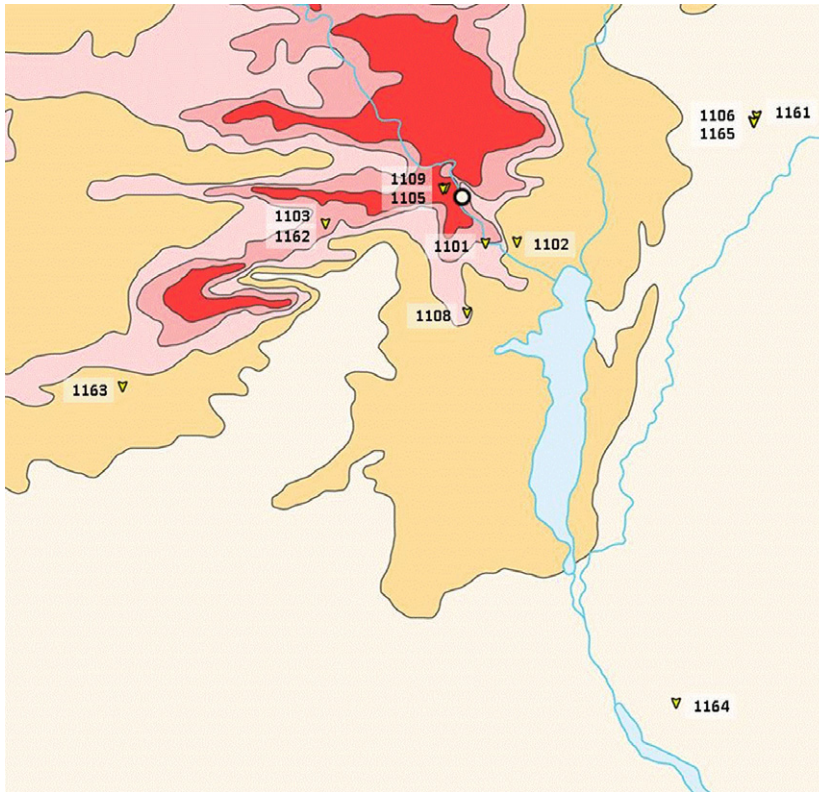
The objectives of this study were to (i) assess anther-smut disease prevalence in *S. latifolia* in relation to pollinator abundance and radiation levels in the field; (ii) estimate deleterious effects of radiation in *M. lychnidis-dioicae* samples from Chernobyl, in terms of spore viability and fertility, karyotype variation and nonsynonymous substitution rates; and (iii) test whether some genes evolve under positive selection specifically in Chernobyl populations. For the second goal of estimating deleterious effects of radiation, we estimated rates of haploid cell viability following meioses from diploid teliospores, karyotypic variation and genomewide ratio of nonsynonymous to synonymous mutations. Mutation rates were estimated by sequencing the genomes of twelve strains from irradiated areas, and the genomes of four strains from the same Central-Eastern European genetic cluster from uncontaminated areas (Vercken *et al.* 2010; Gladieux *et al.* 2011). More specifically, we tested whether dN, dS or dN/dS were significantly higher in contaminated regions and whether a significant correlation between dN, dS or dN/dS and radiation level in the field could be detected. Given the huge effect size of Chernobyl radiation on mutation rates (Møller & Mousseau 2015), elevated mutation rates should be detectable in whole genome sequences.

## Materials and methods

### Data sampling in Chernobyl

We recorded pollinator abundance (butterflies and bumblebees) and anther-smut disease prevalence in *Silene latifolia* in Chernobyl during fieldwork at 16 different sites inside and just outside the Chernobyl exclusion zone during 2010–2015 (Table S1, Supporting information, Fig. 1). We surveyed more than 30 study sites across the Chernobyl exclusion zone and the surroundings. Each study site was checked for the presence of caryophyllaceous plants including *S. latifolia*. When *S. latifolia* was present, the total number of plants and the number of infected plants, that is those with smut spores produced in the anthers, was counted in the immediate vicinity and the number of butterflies and bumblebees seen during an observation period of 5 min at each site was recorded. In large populations of *S. latifolia*, we then moved 100 m to a new site within the *S. latifolia* population where the number of healthy and diseased plants, butterflies and bumblebees were again recorded for 5 min. This process continued until there were no more plants of *S. latifolia* recorded (Møller *et al.* 2012). The abundance of butterflies and bumblebees was subsequently standardized to numbers per





**Fig. 1** Sampling sites of *Microbotryum lychnidis-dioicae* in Chernobyl indicated as triangles. Colour scales from highest (red) to lowest (white) levels of background radiation, corresponding to >40, 15–40, 5–15, 1–15, <1 Ci/km<sup>2</sup>, respectively. The nuclear power plant is represented by a white circle. The scale of the map shows an approximately 200 × 200 km<sup>2</sup> area (Mapping cultural space across Eurasia 2015).

5 min of observation. We collected flower samples in paper bags to bring to the laboratory for further analyses. All surveys and collections were only made on days without rain or strong wind.

#### Postmeiotic viability measures

We spread teliospores of *Microbotryum lychnidis-dioicae* on Potato Dextrose Agar (PDA) plates for 12 strains from the Chernobyl area without prior knowledge of the level of background radiation of the sample (Table S1, Supporting information). On nutritive media in vitro, the fungus undergoes meiosis upon germination of diploid teliospores, and the postmeiotic cells replicate clonally as haploid yeast-like sporidia. On the plant, pairs of sporidia conjugate and produce an infectious hypha. The accumulation of sporidia following spore germination is thus a measure of fertility, in the form of meiotic success and haploid viability, for the diploid individual present in a given flower. After 48 h at 22 °C, the haploid cells derived from spore germination were photographed. In the photographs, we counted the number of sporidia for 100 separate teliospore germinations per sample. Similar counts were performed for three strains from outside the Chernobyl area, but belonging the Central–Eastern European genetic cluster, for comparison with unirradiated areas;

teliospore collections of these strains were the same age as the Chernobyl strains (Table S1, Supporting information).

#### Karyotypes

Haploid cultures were isolated from meiotic tetrads by micromanipulation such that cells of  $a_1$  and  $a_2$  mating types were selected for each of six samples from the Chernobyl region and subjected to pulsed field gel electrophoresis as described previously (Hood *et al.* 2003). Briefly, a CHEF-DR II system (Bio-Rad) was used to generate karyotypes by pulsed field gel electrophoresis using switch times of 200 s (initial) and 1100 s (final) in a 0.8% chromosomal grade agarose for 96 h at 14 °C and 2.7 V/cm. These run conditions optimize visualization of the chromosomes in the genome of *M. lychnidis-dioicae* by separation in the range of 0.90–3.00 million base pairs (mbp). Gel images (stained with SYBR Safe) were acquired by a digital camera.

#### Strains, DNA extraction and sequencing

The genomes of 18 strains of *M. lychnidis-dioicae* collected on *S. latifolia* were sequenced. For this goal, diploid spores from one anther were spread on petri dishes on PDA medium at 23 °C for a few days. A

given flower bears diploid spores from a single individual (Lopez-Villavicencio *et al.* 2007). Therefore, the harvested haploid sporidia on PDA represented thousands of meiotic products of a single diploid individual. Harvested haploid cells were stored at  $-20^{\circ}\text{C}$  until use. DNA was extracted using the Macherey-Nagel NucleoSpin Soil kit #740780.250 following manufacturer's instructions and resuspended in deionized water (100  $\mu\text{L}$ ). DNA purity was assessed by measuring ratio of 230 of 260 and 280 of 260 nm with a NanoDrop 2000 spectrophotometer (Thermo Scientific), and double-stranded DNA concentration was measured with a Qubit 2.0 fluorometer.

Paired-end libraries of  $2 \times 100$  bp fragments with an insert size of 300 bp were prepared with Illumina TruSeq Nano DNA Library Prep Kits, and sequencing was performed on a HiSeq2000 Illumina sequencer, at a depth of coverage of  $100\times$  on average.

We checked that the strains belonged to *M. lychnidis-dioicae* by building a phylogenetic tree using orthologous genes of other *Microbotryum* species; two strains that clustered together in the tree with *Microbotryum* species other than *M. lychnidis-dioicae*, thus representing spillover from other host plant species, were discarded (not shown). The genomes of 16 *M. lychnidis-dioicae* strains were thus retained (Table S1, Supporting information). Of these, the first 12 corresponded to strains sampled in the area of Chernobyl (Fig. 1, hereafter referred to as Chernobyl group), while the remaining four were sampled from different nonradiated locations corresponding to the Central–Eastern genetic cluster in Europe (hereafter referred to as the reference group).

### Sequence analyses

Accession numbers of the genomes of the 16 *M. lychnidis-dioicae* strains analysed are included in Table S1 (Supporting information). The complete genomes (predicted genes and corresponding proteins) of the 16 *M. lychnidis-dioicae* strains and the reference genome (Perlin *et al.* 2015) were used. For each genome, the total collection of predicted gene sequences was used as queries in the OrthoMCL analysis, which implements a sequential bioinformatics pipeline based on blast searches and clustering methods for the prediction of orthologous relationships. The script ORTHOMCL.PL version 1.4 (Li *et al.* 2003) was used with default settings, allowing us to retrieve the full set of shared orthologs that are present as a single copy in all analysed strains (1:1 orthologs). We therefore obtained alignments of alleles among strains.

Initially, OrthoMCL predicted 6145 single-copy orthologous protein-coding genes shared among the 16

analysed strains. However, in 77 cases, no matching protein sequence was found, so all subsequent analyses were conducted with the remaining 6068 genes. For each of these, the predicted genes and the corresponding protein sequences were extracted. First, the protein sequences were aligned and those alignments were subsequently used to guide the predicted gene alignments, taking codons into account, with the PAL2NAL v.14 software (Suyama *et al.* 2006). Next, the program gestimator from the Libsequence library (Thornton 2003) was implemented to obtain all possible pairwise rate estimates of synonymous (dS), non-synonymous (dN) and the corresponding dN/dS ratio, removing those with no synonymous differences (i.e. cases for which dN/dS is estimated to be infinity). To calculate an average dN/dS ratio for each of the 12 Chernobyl strains, we first calculated four independent dN/dS ratios for each strain by comparing it to each of the four reference strains. These four values were then averaged. We calculated the dN/dS ratio of each reference strain compared to the three other reference strains. Diversity estimates were computed using EG-GLIB (de Mita & Siol 2012). We checked using a larger genome data set (T. Giraud, H. Badouin & G. Aguilera, unpublished data) that all the reference strains belonged to the genetic Central–Eastern European cluster as previously identified based on microsatellite data (Vercken *et al.* 2010) and that no further population subdivision was found within this cluster (not shown).

To examine the capacity for melanin production, genes involved in three melanin pathways in fungi were examined: the DHN-melanin (Wheeler *et al.* 2008), DOPA melanin (Langfelder *et al.* 2003) and L-tyrosine degradation (Schmaler-Ripcke *et al.* 2009; Keller *et al.* 2011). Genes for each pathway in *M. lychnidis-dioicae* were identified by identifying orthologs using OrthoMCL as above with the gene set of *Aspergillus niger*. Whenever orthologs were not identified, the most similar sequences based on blastp were examined; in all cases, this did not identify any additional orthologs, *that is* for the multicopper oxidases (MCOs) involved in DHN-melanin synthesis this identified only ascomycete laccase MCOs.

### Formal statistical tests for detecting dN/dS variability and positive selection

In order to assess the statistical support for differences in dN/dS between strains from Chernobyl and reference strains, we used the CODEML program (Yang 2007) in the PAML package to obtain the nonsynonymous to synonymous substitution ratio (dN/dS). For these tests, a tree of the strains was reconstructed, using the Italian

reference genome as the outgroup (Badouin *et al.* 2015; Perlin *et al.* 2015). First, we tested whether each gene evolved with different dN/dS rates in Chernobyl strains compared to other strains. A branch-specific analysis was thus used to compare two models representing different dN/dS variability patterns: the null model assumed that all branches in the tree evolved under the same dN/dS ratio and the alternative model assumed that the branches grouping the Chernobyl strains were subject to a different dN/dS ratio from those grouping the Eastern reference strains. We compared the two models with a likelihood ratio test (LRT), with twice the difference in the log likelihood score of the two models being approximated to a chi-square distribution and degrees of freedom equal to the difference in model parameters.

A second test was performed to identify genes under positive selection in strains from Chernobyl. In this test, we used the genes that were found in the first test to evolve with significantly higher dN/dS rates in strains from Chernobyl compared to other strains, and with the largest differences. Two nested models, M1a and M2a, were compared by means of a LRT. Model M1a assumed that the sites in the alignment fell into either of two classes of sites, one where dN/dS can take values between 1 and 0 and another class of sites where dN/dS is fixed at 1. On the other hand, model M2a allows for an extra class of sites where dN/dS is allowed to take values >1, thereby identifying the sites that may have evolved positive selection. A LRT was used to compare the two nested likelihood models, as described.

### Statistical analyses

ANOVA, mean comparisons and correlations were performed using JMP (SAS Institute) and power analysis using the Z transformation method (Lachin 1981) at <http://www.cct.cuhk.edu.hk/stat/other/correlation.htm>. Radiation and abundance data were log-transformed to improve the normality of their distributions.

## Results

### Prevalence data of the disease

Among the 30 study sites surveyed across the Chernobyl exclusion zone (Fig. 1) and the surroundings, there were 14 sites, all having high levels of background radiation, where no *Silene latifolia* plants were recorded. In the sites with *S. latifolia* plants, we explored the effect of the abundance of butterflies, bumblebees and radiation level on anther-smut disease prevalence in *S. latifolia* (Table 1). Disease prevalence increased with pollinator abundance and significantly so with log-transformed butterfly abundance [Fig. 2; analysis weighted by sample size:  $F_{1,19} = 15.15$ ,  $r^2 = 0.41$ ,  $P = 0.001$ , slope (SE) = 0.426 (0.109)]. Log-transformed butterfly abundance decreased with log radiation levels [Fig. 3; analysis weighted by sample size:  $F_{1,19} = 15.48$ ,  $r^2 = 0.42$ ,  $P = 0.0009$ , slope (SE) = -0.107 (0.027)]. Therefore, we included pollinator abundance (numbers of both butterflies and bumblebees) in the model testing for an effect of radiation levels on disease prevalence and carried out a stepwise model construction procedure to minimize the AIC criterion, weighting by total sample size. The best model included only radiation level and butterfly abundance and their interaction. Disease prevalence significantly decreased with radiation level (Table 1, Fig. 4), but did not change significantly with the abundance of butterflies. However, we found a significant interaction, such that disease prevalence increased with butterfly abundance at low radiation levels but decreased somewhat with increasing butterfly abundance at high-radiation levels (Fig. 5). Similarly, disease prevalence decreased strongly with increasing radiation levels, but only at high butterfly abundance (Table 1, Fig. 5). Not only the prevalence but also the number of infected plants significantly decreased with the level of radiation ( $r = -0.58$ , d.f. = 29,  $P = 0.0011$ ), indicating lower population size for *Microbotryum*

**Table 1** Factorial ANOVA analysis, weighted by sample size, of the effect of the abundance of butterflies and radiation level ( $\mu\text{Gy/h}$ ) on disease prevalence (all explanatory variables log-transformed). An initial model including bumblebee abundance and all interactions was constructed and reduced using a stepwise procedure with minimum AIC criterion. The model statistics are  $F_{3,17} = 15.316$ ,  $r^2 = 0.68$ ,  $P < 0.0001$ . The VIF (variance inflation factor) quantifies the degree of collinearity in the model, which here is low and can thus be discounted

	d.f.	Sum of squares	F	P	Estimate (SE)	VIF
Intercept			4.452	0.05	0.097 (0.046)	
Log radiation	1	1.665	8.582	0.009	-0.052 (0.017)	1.899
Log abundance butterflies	1	3.167	1.633	0.22	0.145 (0.114)	1.983
Log radiation x log butterflies	1	2.543	13.105	0.002	-0.462 (0.128)	1.093
Error	17	3.298				



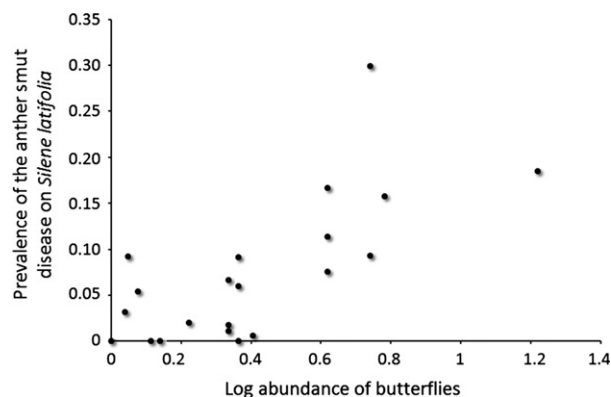


Fig. 2 Prevalence of the anther-smut disease on *Silene latifolia* plotted against butterfly abundance (log-transformed).

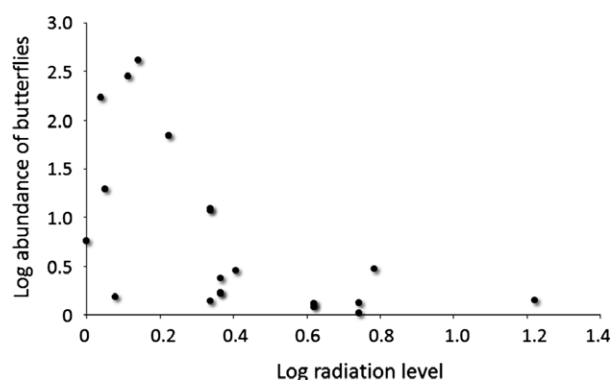


Fig. 3 Butterfly abundance plotted against radiation level ( $\mu\text{Gy/h}$ ), both log-transformed.

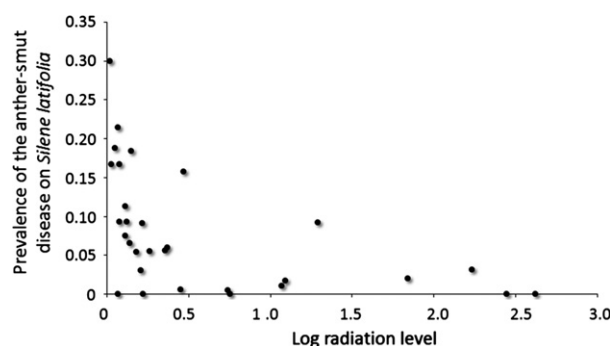


Fig. 4 Prevalence of the anther-smut disease on *Silene latifolia* plotted against the radiation ( $\mu\text{Gy/h}$ ).

*lychnidis-dioicae*, which may lessen the efficacy of natural selection.

#### Postmeiotic viability measures

Fertility measures in the form of meiotic success and haploid viability following spore germination, assessed

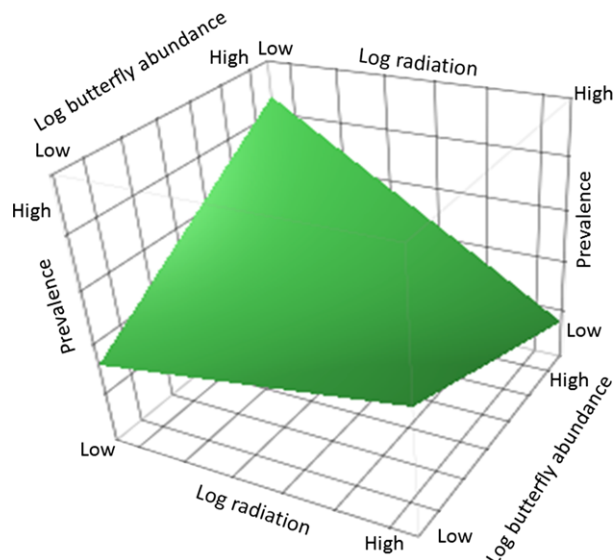


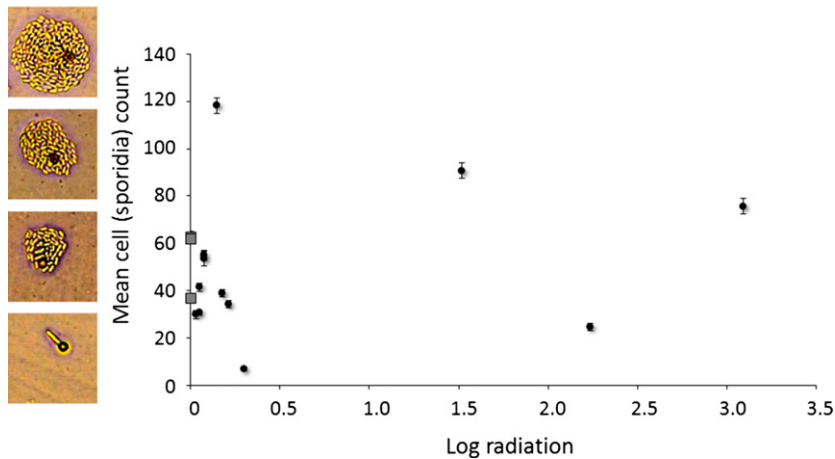
Fig. 5 Disease prevalence as a function of radiation level ( $\mu\text{Gy/h}$ ) and butterfly abundance (all log-transformed).

for 12 *M. lychnidis-dioicae* samples from the Chernobyl area (Table S1, Supporting information, Fig. 6), showed no significant correlation between cells counted (log-transformed) and radiation level at the source of the samples ( $r = 0.19$ ,  $n = 12$ ,  $P = 0.55$ , Fig. 6). With this weak positive correlation coefficient, a sample size of more than 105 strains would be required to detect a significant effect of radiation level. There was no significant difference in the average postmeiotic cell number between strains from radiated and nonradiated areas (Table S1, Supporting information;  $t_{13} = 2.18$ ,  $P = 0.82$ ; Fig. 6). The fertility measures suggested high viability for all samples except from location 1161 (Table 1, Supporting information), which has less than seven cells per germination after 48 h of incubation, indicating either the failure of meiosis or the inability of the four meiotic products to complete a single mitotic division of the yeast-like cells. All other samples had cell counts indicating multiple rounds of mitotic divisions in the same time period.

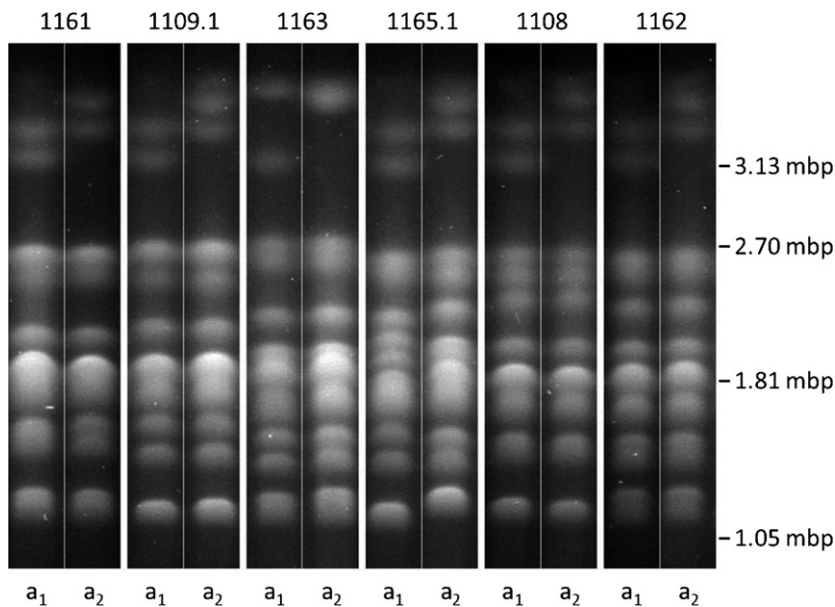
#### Karyotypes

Chromosome profiles of samples from the Chernobyl region (Fig. 7) showed no abnormalities that would be distinct from prior karyotype studies of this species (Hood 2002; Hood *et al.* 2003; Hood & Antonovics 2004). The distribution of chromosome sizes ranged from 1 to 4 Mbp, with chromosome numbers of ca. 12 per haploid genome. The genomes contained the large dimorphic mating-type chromosomes (ca. 3.3–4 Mbp) that have been shown to occur throughout the species (Hood 2002). In the size, range of the





**Fig. 6** Fertility and viability for *Microbotryum lychnidis-dioicae* samples from the Chernobyl area as black dots and for reference strains from uncontaminated regions as grey squares, plotted against log radiation level ( $\mu\text{Gy/h}$ ). Fertility and viability are estimated as the mean of postmeiotic haploid sporidia numbers ( $\pm$  standard error) growing from each of 100 diploid teliospores. At left, some pictures used for counting sporidia.



**Fig. 7** Karyotypes of haploid cell cultures of opposite mating types ( $a_1$  and  $a_2$ , respectively) from one postmeiotic tetrad for each of six samples of *Microbotryum lychnidis-dioicae* from the Chernobyl area (see Table S1, Supporting information for details on the samples). Chromosome assessment was performed by the segregation of chromosome size variation in the  $a_1/a_2$  pairs of electrophoretic karyotypes. Chromosome size is given in mega base pairs based upon the commercial size standards (*Hansenula wingei*; Bio-Rad). The dimorphic chromosomes at the top of the gel are the  $a_1$  and  $a_2$  mating-type chromosomes (Hood 2002; Hood *et al.* 2013).

mating-type chromosomes is a large autosome, while the other autosomes span sizes below 2.6 Mbp. The  $a_1$  and  $a_2$  pairs of haploid genomes displayed karyotypes with high levels of autosome homomorphism (equally sized homologues segregating in meiosis), even in samples such as 1161 with the poorest post-meiotic growth ability. Some minor variability among strains and some autosomal size heteromorphism were observed between karyotypes (with some dimorphic homologous chromosomes segregating in meiosis, Fig. 7). However, this was within the range of previously quantified variation in *M. lychnidis-dioicae* in unirradiated areas including two sample locations from the Eastern cluster that were also examined here for most meiotic fertility (DA00 and CZ00 in Table S1 (Supporting information); Hood & Antonovics 2004).

#### Genome sequences

A total of 6068 predicted genes corresponding to  $1.04 \times 10^7$  base pairs (bp) were analysed, with an average of  $1.71 \times 10^3$  bp per gene alignment. Of these base pairs, a total of  $1.94 \times 10^5$  corresponded to polymorphic sites, with an average of 83.28 polymorphic sites per analysed gene alignment. Other diversity indicators yielded averages per site for  $\pi$  of 0.0010 for Chernobyl strains and 0.0017 for the reference strains, and for  $\theta$  of 0.0005 for Chernobyl strains and 0.0008 for the reference strains.

We detected no significant correlation between either synonymous or nonsynonymous substitution rates and the radiation measurements at the various sampling sites in Chernobyl (for dS,  $r = 0.25$ ,  $n = 12$ ,  $P = 0.44$ , Fig. S1 (Supporting information) for dN,  $r = 0.37$ ,

$n = 12$ ,  $P = 0.23$ , Fig. S2, Supporting information). The average pairwise dN/dS ratios ranged between 0.336 and 0.359. We detected no sign of genomic degeneration in the Chernobyl area from the nonsynonymous substitution rates. Indeed, we found no significant correlation between the average pairwise dN/dS values and the radiation measurements at the different sampling sites in Chernobyl ( $r = 0.068$ ,  $n = 12$ ,  $P = 0.83$ , Fig. 8, Table S1, Supporting information). With this correlation, a sample size of more than 830 genomes would be required to detect a significant weak correlation. The mean dN/dS values were even significantly lower in the Chernobyl strains (mean dN/dS = 0.346) than in the reference group (mean dN/dS = 0.353) (Student's  $t$ -test,  $t = 2.16$ , d.f. = 14,  $P = 0.004$ ; Fig. 8).

Formal tests using PAML confirmed these results. Out of 6068 individually tested gene alignments, 575 showed significant differences in their dN/dS rates between Chernobyl strains and other strains. Among these, most (441, i.e. 77%) had a lower dN/dS in Chernobyl strains than in other strains.

The 15 genes with the highest ratio of dN/dS in Chernobyl strains compared to other strains were further analysed with a site-specific test looking for genes that would evolve under positive selection in Chernobyl strains. Indicating positive selection at these genes in Chernobyl strains. Two of these five genes had assigned putative functions, one involved in cell division and another in protein degradation.

Because we found no deleterious effect of radiation on *M. lychnidis-dioicae* and considering that melanin has frequently been invoked as a mechanism of radio resistance in bacteria and fungi (Dadachova *et al.* 2007; Khajo *et al.* 2011; Tugay *et al.* 2011), we searched for homologs to genes known to be involved in melanin synthesis in genome sequences. Very few homologs to genes involved in the melanin biosynthesis pathway

could be found in the *M. lychnidis-dioicae* genome (Table 2).

## Discussion

Environmental levels of ionizing radiation are shown here to have a strong albeit complex influence on the distribution of a fungal plant pathogen that may be mediated by alterations of the larger biological community. The prevalence of anther-smut disease caused by the fungus *M. lychnidis-dioicae* was indeed much lower in more contaminated areas, likely influenced by an interaction with pollinators, in particular butterflies. Indeed, in areas with little radioactive contamination, anther-smut prevalence increased with abundance of butterflies, as expected given that spores are transmitted by pollinators (Roche *et al.* 1995; Altizer *et al.* 1998). At highly contaminated sites, the opposite pattern was found, with disease prevalence decreasing slightly with greater butterfly abundance. Indeed, the parameter set leading to the lowest disease prevalence was highest radiation coupled with highest butterfly abundance, suggesting that at very contaminated sites even high opportunity for spore transmission results in little infection. The stress associated with high-radiation levels may increase resistance to this pathogen in plants, as abiotic and biotic stresses may interact in complex ways, in some cases one enhancing resistance to the other (Atkinson & Urwin 2012). However, previous studies have found low overall resistance of plants to biotic stress in Chernobyl (Dmitriev *et al.* 2011). Alternatively, changes in developmental or behavioural traits of the organisms in high-radiation environments could have a large influence on the dynamics of disease transmission. In particular, butterflies could be poorer spore vectors at high-radiation level, as previous studies of birds, spiders, plants and insects have shown more behavioural abnormalities at higher radiation levels (Møller & Mousseau 2013). If the hosts' flowering behaviour is altered at high-radiation levels, it may avoid the disease because plants that shed their flowers more rapidly become infected less often despite receiving infectious spores (Kaltz & Shykoff 2001). Further investigations should pursue the individual-level variation in traits of the host and pollinators that are likely to impact disease transmission rates.

We found no evidence of genomic or genetic changes in the fungal pathogen that may have been expected under conditions of high ionizing radiation levels. For karyotypes, fertility in terms of meiotic success, viability of the haploid stage, or frequencies of nucleotide or amino-acid substitutions, there was no evidence of deleterious effects on *M. lychnidis-dioicae* as a function of radiation level at the site of

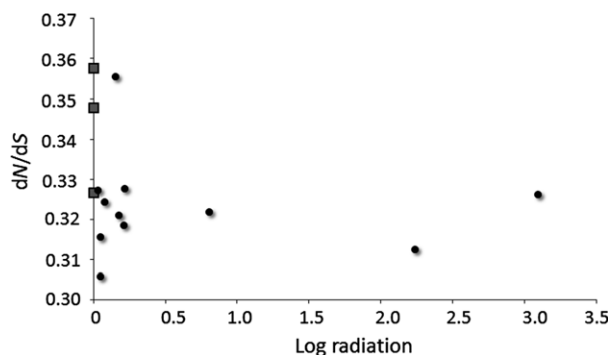


Fig. 8 Mean nonsynonymous substitution rate over synonymous substitution rate (dN/dS) for *Microbotryum lychnidis-dioicae* strains from the Chernobyl area as black dots and for reference strains from uncontaminated regions as grey squares, plotted against log radiation level.

**Table 2** Orthologs of the three main melanin synthesis pathways detected in the genomes of *Aspergillus fumigatus*, *Aspergillus niger* and *Microbotryum lychnidis-dioicae*

Gene name	<i>A. fumigatus</i> *	<i>A. niger</i>	<i>M. lychnidis-dioicae</i>	Description
DHN-melanin pathway				
Abr2	<b>Afu2g17530 (Abr2)</b> Afu1g15670 Afu4g14280	An01g13660 (McoB) An01g14010 (McoA) An03g03750 (McoC) An04g10400 (McoO) An05g02540 (McoP) An14g05370 (BrnA) An01g11120 (McoE)	No ortholog No ortholog No ortholog No ortholog No ortholog No ortholog	Fungal pigment MCO
Abr1	<b>Afu2g17540 (Abr1)</b> <b>Afu5g03790 (FetC)</b>	An01g08960 (McoH) An15g05520 (McoK)	MVLG_01868	Fungal ferroxidase
Ayg1	<b>Afu2g17550 (Ayg1)</b>	An14g05350 (Ayg1)	No ortholog	1,3,6,8-Tetrahydroxynaphthalene reductase
Arp2	<b>Afu2g17560 (Arp2)</b>	An02g00220	No ortholog	
Arp1	<b>Afu2g17580 (Arp1)</b> <b>Afu2g17600 (Pks1)</b>	An08g09920 An03g05440	No ortholog No ortholog	Scytalone dehydratase
PKS1	Afu4g00210 (EncA) Afu4g14560 Afu7g00160	An04g09530 An09g05730 (FwnA) An11g07310	No ortholog No ortholog No ortholog	Polyketide synthase
DOPA melanin pathway				
melC2	Afu3g01070	An01g09220 (MelC2) An03g00280	No ortholog No ortholog	Tyrosinase
melO		An12g01670 An09g02980 An12g05810 (McoJ) An16g02020 (McoM) An11g03580 (McoD) An08g08450 (McoG) An05g02340 (McoF) An01g00860 (McoN) An18g02690 (McoI)	No ortholog No ortholog No ortholog MVLG_00670, MVLG_02184, MVLG_03092	
	Afu4g14490			Laccase
L-Tyrosine degradation pathway				
Tat	Afu2g13630	An02g05540	MVLG_06370	Tyrosine aminotransferase
hpdD	Afu2g04200	An11g02200	No ortholog	4-Hydroxyphenylpyruvate dioxygenase
hmgA	Afu2g04220	An11g02180	No ortholog	Homogentisate dioxygenase
fahA	Afu2g04230	An11g02170	MVLG_02428	Fumarylacetoacetate hydrolase
maiA	Afu2g04240	An11g02160	No ortholog	Maleylacetoacetate isomerase

\**Aspergillus fumigatus* genes that belong to the DHN-melanin gene cluster (Tsai *et al.* 1999) are in bold; other genes share sequence similarity with the DHN-melanin genes.

collection. Our analyses suggested that this was not due to a low statistical power. The magnitude of the correlation observed here ( $r = 0.07$ ) was an order of magnitude below the mean estimate for effects of ionizing radiation on mutations at Chernobyl from a meta-analysis of 30 plant and animal species ( $r = 0.67$ , 95% confidence intervals 0.59–0.73, Møller & Mousseau 2015). We even found lower mean values of dN/dS in Chernobyl, which may be due to stronger selection in contaminated areas against individuals bearing mildly deleterious mutations, as previous studies have found evidence for more intense selection against inferior phenotypes in Chernobyl (i.e.

stronger purifying selection) (Møller 1993, 2002; Ellegren *et al.* 1997; Møller & Mousseau 2001, 2003, 2016; Møller *et al.* 2005a,b, 2012, 2013).

The lower abundance of pollinators in contaminated areas is unlikely to have biased dN/dS values by inducing higher selfing rates. Indeed, *M. lychnidis-dioicae* is highly automictic (Giraud *et al.* 2008), so the low disease prevalence in Chernobyl is unlikely to lead to increased selfing rates, which could result in higher accumulation of deleterious mutations in itself. In any case, this effect should lead to an increase in the dN/dS values in contaminated areas, while we observed the opposite.

Melanin has frequently been invoked as a mechanism of radio resistance in bacteria and fungi (Dadachova *et al.* 2007; Khajo *et al.* 2011; Tugay *et al.* 2011), but a broader survey of radiation effects on melanin-free microbes from natural communities that may reveal alternative resistance mechanisms is lacking from the literature. The presence of melanin is, as yet, unreported in *Microbotryum*, and very few homologs to genes known to be involved in melanin synthesis could be found in its genome. However, red pigments have been described in *M. lychnidis-dioicae* (which was named *Ustilago violacea* at that time; Will *et al.* 1984; Will & Reppe 1984). Some other red-pigmented organisms are radio resistant (Asker *et al.* 2007; Yuan *et al.* 2009; Copeland *et al.* 2012; Su *et al.* 2014), although red pigmentation is more generally considered as protection against UV radiation, including in *M. lychnidis-dioicae* (Will & Reppe 1984; Will *et al.* 1984). Possibly, red pigmentation that had been selected to confer UV resistance also implies radio resistance, a hypothesis that should be explored in future studies.

A number of studies have found evidence of adaptation to low-dose radiation at Chernobyl, particularly in fungi. These range from proteomic analyses (Danchenko *et al.* 2009; Klubicova *et al.* 2010) and studies of DNA methylation (Kovalchuk *et al.* 2003) to other physiological mechanisms (Kovalchuk *et al.* 2004; Klubicova *et al.* 2012). There is also evidence consistent with adaptation through the intracellular antioxidant glutathione (GSH; Galván *et al.* 2014). Perhaps the most clear-cut evidence of adaptation concerns resistance to radioactivity in generalist bacteria that are widely distributed across Europe (Ruiz-González *et al.* 2016).

In conclusion, our study reinforces the view that fungi, even nonmelanized species, can cope well with the potential direct effects of high-radiation levels, while indirect effects mediated through biological interactions (host plants and pollinators) may limit pathogen distributions. A previous study reported the emergence of a more virulent crop pathogen population in Chernobyl, and this could be a consequence of selection for resistance to radiation (Dmitriev *et al.* 2011), although reduced host plant fitness may also play a role. Finally, our findings also suggest the existence of strong purifying selection in radiated areas, and possibly positive selection on some pathways involved in cell division and abnormal protein degradation.

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- T.G. and A.P.M. designed research; A.P.M. and T.M. collected data and strains in Chernobyl; G.A. and H.B. analysed the genomes; H.B., S.L.P., T.G. and A.S. performed strain cultivation and DNA extractions; S.G. and C.A.C. helped with genome analyses; M.E.H. performed experiments of viability and fertility and of karyotypes; T.G., A.P.M. and J.A.S. analysed data; T.G. and A.P.M. wrote the study with contributions by all authors.

## Data accessibility

Data and genome Accession nos are provided in Table S1 (Supporting information).

## Supporting information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Mean synonymous substitution rate (dS) for *Microbotryum lychnidis-dioicae* strains from the Chernobyl area, plotted against log radiation level ( $\mu\text{Gy/h}$ ).

**Fig. S2** Mean non-synonymous substitution rate (dN) for *Microbotryum lychnidis-dioicae* strains from the Chernobyl area, plotted against log radiation level ( $\mu\text{Gy/h}$ ).

**Table S1** Information on *Microbotryum lychnidis-dioicae* strains for which genomes have been analysed: strain ID, GPS coordinates of the nearest town, prevalence of the disease in the population, date of collection, spore viability and fertility (mean number of haploid sporidia germinating from 100 diploid teliospores after 48 h), radiation level measure ( $\mu\text{Sv/h}$ ) at the very collection site (radiation level vary at distances of 10 m) and mean non-synonymous substitution rate over synonymous substitution rates (dN/dS) in its genome compared to reference strains from uncontaminated areas belonging to the same Eastern genetic cluster.